

Prognostic importance of antigen-presenting dendritic cells during vaccine therapy in a murine hepatitis B virus carrier

SK. MD. F. AKBAR, N. HORIIKE & M. ONJI *Third Department of Internal Medicine, Ehime University School of Medicine, Ehime, Japan*

SUMMARY

As, the outcome of vaccine therapy was extremely heterogeneous in both human and murine hepatitis B virus (HBV)-carriers, the experiments presented here were performed to find out a prognostic marker of vaccine therapy using an animal model of HBV-carrier state, HBV-transgenic mice (Tg). Neither the prevaccinated titres of viral markers, such as hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) or HBV DNA, nor the function of lymphocytes prior to vaccination, had significant influence on the outcome of vaccine therapy. Two independent, placebo-controlled, trials of vaccine therapy for 12 months, one in 17 HBV-Tg and the other in 26 HBV-Tg (total, $n=43$) showed that the eight of 17 and 15 of 26 HBV-Tg that had potent dendritic cell (DC) function at the start of vaccine therapy became completely negative for HBsAg, HBeAg and reduced HBV DNA, whereas all 19 HBV-Tg that had poor DC function at the start of vaccine therapy became complete non-responders, although, the prevaccinated titres of HBsAg, and HBeAg were similar in all 43 HBV-Tg. Further study to find the mechanism underlying this revealed that there was up-regulation of major histocompatibility complex (MHC) class II, CD86 antigens on DC and increased production of interleukin-12 (IL-12) by DC and of IL-2, and tumour necrosis factor- α (TNF- α) in DC/T-cell cultures when vaccine containing HBsAg was injected in HBV-Tg with potent DC function but not in HBV-Tg with poor DC function. This is the first report on the prognostic importance of DC during an immune therapy. Degree of activation of DC following vaccination would *possibly* help to predict the outcome of vaccine therapy in human HBV-carriers. These data also provide the scientific and logical basis to up-regulate the function of the DC before an immune therapy.

INTRODUCTION

Hepatitis B virus (HBV) is a non-cytopathic virus and causes chronic liver diseases including cirrhosis of liver and hepatocellular carcinoma.¹ Both the virus and the immune response of the host play a major role in the pathogenesis of persistent HBV infection. Like other chronic infections, treatment of

HBV-carriers is difficult and time consuming.² Interferon is recommended for therapy in HBV-carriers and initially only one-third of the HBV-carriers have benefited from interferon therapy. But, extensive clinical trials showed prognostic markers of interferon therapy and now more than 70% of HBV-carriers with good prognostic markers [hepatitis B e antigen (HBeAg) positivity and moderate degree of DNA polymerase activities] show long-term remission following interferon therapy,^{3–5} indicating the importance of prognostic markers in the clinical set up.

Recently, a potent immunotherapy, called vaccine therapy, has been proposed for HBV-carriers, in which injections with vaccine containing hepatitis B surface antigen (HBsAg) alone or with other HBV-related proteins have shown both antiviral and immunomodulatory effects in HBV-carriers.⁶ One study of vaccine therapy in 32 human HBV-carriers has shown complete clearance and reduction of HBV DNA in 10 and four HBV-carriers, respectively,⁷ on the other hand, a second trial in 14 HBV-carriers reported clearance of HBV DNA in nine, HBeAg in six and development of antibody to HBeAg in two HBV-carriers.⁸ Although, these clinical trials have shown the initial promise of this new immunotherapy, nothing

Received 23 June 1998; revised 21 September 1998; accepted 24 September 1998.

Abbreviations: anti-HBs, antibody to hepatitis B surface antigen; APC, antigen-presenting cell; C, complement; Con A, concanavalin A; DTgU (1:0), amounts of anti-KLH IgG in 1:80 000 dilution of standard sera; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate conjugate; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBV-Tg, hepatitis B virus transgenic mice; i.p., intraperitoneally; KLH, keyhole limpet haemocyanin; LPS, lipopolysaccharides. MLR, mixed leucocyte reaction; PBS, phosphate-buffered saline; OD, optical density; SD, standard deviation.

Correspondence: Sk. Md. F. Akbar, Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-Cho, Ehime 791-0295, Japan.

is known as to why only some of the HBV-carriers responded to vaccine therapy, while others did not. Again, nothing is known regarding the virus-related or host-derived factors that might be useful to predict the outcome of this therapy.

Vaccine therapy is a type of immune therapy and it is postulated that both viral and/or host derived-factors and their interactions are vital to predict the outcome of this therapy. But there is an obvious limitation to the performance of elaborate studies regarding host-virus interactions in humans due to major histocompatibility complex (MHC) mismatching among individuals.

We and others have shown that HBV transgenic mice (HBV-Tg)⁹⁻¹¹ is a useful animal model of HBV-carrier state to elucidate the viral biology and to study the host-virus interactions, when these are used to address questions that cannot be otherwise approached by the existing methodology in human HBV-carriers. To investigate the utility of HBV-Tg as an animal model of the HBV-carrier state during vaccine therapy, we conducted a double-blind, placebo-controlled trial of vaccine therapy in a group of 32 HBV-Tg.¹² Similar to human HBV-carriers, the vaccine therapy was effective in murine HBV-carriers but the outcome was heterogeneous and the titres of HBV-markers were not useful to predict the outcome of vaccine therapy; we postulated that the host-derived factor(s) might have influenced the outcome of vaccine therapy in both human and murine HBV-carriers.

The present communication has described a series of experiments in HBV-Tg to find out a prognostic marker of vaccine therapy. First, a trial vaccine therapy was conducted in a group of HBV-Tg for 12 months, which showed that some HBV-Tg responded to vaccine therapy, whereas, others became non-responders, although, the prevaccinated levels of HBsAg, HBeAg and HBV DNA were similar in all HBV-Tg.

Further experiments revealed that although the function of lymphocytes was similar, the stimulatory capacity of DC, the most potent antigen-presenting cell (APC), was heterogeneous among HBV-Tg; we speculated that the function of DC might have prognostic importance during vaccine therapy. However, there was an obvious limitation to the study of DC function at the start of vaccine therapy and the evaluation of the response to vaccine therapy in the same HBV-Tg after 12 months, because DC were usually isolated after killing the HBV-Tg. This was overcome when a good correlation was established between the serum titres of keyhole limpet haemocyanin (KLH)-specific antibody following immunization with a specific dose of KLH and the stimulatory capacity of DC in both untreated and vaccinated HBV-Tg. Based on these findings, two trials of monthly vaccine therapy were conducted in HBV-Tg for 12 months to see whether the function of DC before the start of therapy had any influence on the final outcome of vaccine therapy. We have also studied why only HBV-Tg with potent DC function responded to vaccine therapy and the relationship between activation of DC and therapeutic activity of vaccine therapy has been discussed.

MATERIALS AND METHODS

Animals

HBV-Tg (official designation: 1.2HB-BS10) were produced by microinjecting a partial tandem duplication of the complete HBV genome (Subtype Adr) into fertilized eggs of C57BL/6

mice. HBV-Tg produced HBsAg, HBeAg and HBV DNA in sera and HBV-related mRNAs were expressed in liver, kidney and testis.⁹ Normal C57BL/6 (H-2^b) and C3H/He (H-2^k) mice (Charles River INC, Nagoya, Japan) were used in mixed cultures and allogenic mixed leucocyte reaction (MLR), respectively. HBV-Tg and normal mice were kept separately and bred in our animal facility. All animals received humane care according to the institutional guidelines.

Reagents and antibodies

RPMI-1640 with 10% heat inactivated fetal calf serum was used as culture medium, unless specified otherwise. Collagenase III was used to make a single-cell suspension of spleen (Sigma, St. Louis, MO). Cytotoxicity medium containing 0.3% bovine plasma albumin and 25 mM HEPES in RPMI-1640 was obtained from Cedarlane Lab. Ltd. (Hornby, Ontario, Canada). Viable cells were separated from dead cells by applying on a separation gradient, Lympholyte-M (Ficoll 400 and sodium diatrizoate, density 1.0875 ± 0.00005 , Cedarlane).

The antibodies used in this experiment have been described in detail in previous reports.^{13,14} In short, monoclonal antibodies to Thy-1.2 (clone 5a-8), Lys-1.2 (clone CG16) (Cedarlane, Ontario, Canada), and CD45R (clone RA-3-6B2) (Pharmingen, San Diego, CA) were used to deplete T cells, and B cells, respectively, with low-toxic complement (C) (Cedarlane). Hamster anti-mouse CD11c (N418), a DC-specific antibody¹⁵ and fluorescein isothiocyanate (FITC)-conjugated antibody to hamster IgG (Cappel, Malvern, PA) were used to stain cells in flow cytometry. FITC-conjugated antibody to mouse Ia (clone M5/114) (Boehringer Mannheim Biochemica, Mannheim, Germany), FITC-conjugated antibody to mouse CD86 (B 7-2), and FITC-conjugated antibody to mouse CD80 (B 7-1) (Pharmingen) were used to study the expression of these antigens on DC.

Detection of HBV-related antigens and antibody

HBsAg, HBeAg and antibody to HBsAg (anti-HBs) in sera were detected by the haemagglutination method, as described using commercial kits (Mycell, Tokyo Institute of Immunology, Tokyo, Japan).¹² In these methods, sera showing haemagglutination at a dilution $>2^2$ were positive for respective HBV-markers.

Detection of serum HBV DNA by polymerase chain reaction (PCR)

Total DNA were isolated from sera exactly according to an already described method¹² and were adjusted to different concentrations between 50.0 ng and 0.00005 ng/ μ l by 10-fold serial dilution. The HBV DNA sequence in the samples was amplified by PCR with synthetic oligonucleotides spanning X/precore/core region of HBV DNA. For 1.0 μ l of total DNA, 25 pmol sense primer [nucleotides (nt) 1725-1747, AAGGACTGGGAGGAGTTGGGGGA], 25 pmol anti-sense primer (nt 1966-1946, GAGAGAAAAACGGAAGACTG), 2.5 mmol dATP, dCTP, dGTP and dTTP each, $10 \times$ TPCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl) and 2.5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) were adjusted in 46.0 μ l of total volume. After a 5-min denaturing step at 94°, 35 cycles, each lasting 60 seconds at 94°, 90 seconds at 50°, and 150 seconds at 72° were run. Elongation was completed by an additional 7-min incubation at 72°. The

PCR product was visualized electrophoretically in an ethidium-bromide-stained 3% agarose gel. The presence of a band 242 base pairs (bp) long was considered a HBV DNA-specific signal. Negative control consisted of serum DNA from normal C57BL/6 mice.

In situ hybridization for HBV DNA in liver

In situ hybridization for HBV DNA was performed using the *in situ* hybridization kit using a non-radioactive digoxigenin-labelled DNA probe (Kreatech Biochemistry, Amsterdam, the Netherlands), as described.¹⁴ In short, paraffin-embedded liver specimens were cut in 4–6- μ m sections, heat-fixed at 56° for 16 hr, deparaffinized in xylene, digested in pepsin (0.01 M HCl, 1:80), prehybridized in prehybridization mixture, supplied with the kit and then digoxigenin-labelled HBV DNA probe was applied to the specimen, and denatured at 90°. Finally, the staining was completed by incubating with alkaline phosphatase-labelled, antidigoxigenin, and subsequently with nitro-blue tetrazolium and 5-bromo-4-chloro-indolyl-phosphate. Positive signals for HBV DNA were detected as blackish granules in the liver. Controls were stained with a negative control probe supplied with the kit. Moreover, livers from normal C57BL/6 mice were stained with a digoxigenin-labelled HBV DNA probe to use as controls.

Immunization with KLH

When mice were injected with KLH only, they received two injections of KLH, intraperitoneally (i.p.), in phosphate-buffered saline (PBS) at an interval of 2 weeks. In most of the experiments, HBV-Tg were injected with 5 μ g of KLH, i.p. in PBS and boosted once after two weeks.

Detection of anti-KLH IgG and non-specific IgM and IgG

KLH-specific and LPS-induced non-specific IgM and IgG productions were determined by an enzyme-linked immunosorbent assay (ELISA) according to the method already described.^{13,14} In short, binding capacities of peroxidase-conjugated goat anti-mouse IgG were assayed as enzymatic reaction of hydrolysis of a substrate, orthophenylenediamine, by measuring the optical density (OD) value at 492 nm with an ELISA reader (Sjeia Auto Reader, Model ER-8000, Sanko Junyaku Co. Ltd, Tokyo, Japan). Pooled sera from unprimed mice were taken as negative control and sera containing known amounts of IgM and IgG (Binding Site Limited, Birmingham, UK), and standard sera rich in anti-KLH IgG were used as positive control. In the anti-KLH IgG assay, the cut-off value for a positive response was the mean \pm three standard deviations (SD) of the OD value for the negative control. Since the OD values at 1:80 000 of anti-KLH IgG-rich standard sera were almost equal to the cut-off value, their OD value was designated as 1.0 DTgU. For all practical purposes, anti-KLH IgG levels \geq 1.0 DTgU were considered a positive anti-KLH IgG response. Levels of anti-KLH IgG in specimen were estimated based on the standard curve plotting OD values at different dilutions of anti-KLH IgG-rich standard sera.

Vaccine therapy in HBV-Tg

Male HBV-Tg, aged 6–8 weeks, and positive for HBV DNA in sera and having titres of HBsAg and HBeAg of 2⁷ and 2⁴ in sera, respectively, were randomly assigned to receive i.p., injection of complete Freund's adjuvant (CFA) only (placebo

recipient) or HBV-vaccine containing 10.0 μ g of HBsAg (subtype; Adr, Research Foundation for Microbial Diseases of Osaka University, Osaka, Japan), emulsified in CFA, i.p. at entry and once in a month for consecutive 12 months (vaccine-recipient) or left untreated (control). Sera were collected from all HBV-Tg before the start of the experiment and were the mice were bled monthly for the placebo and vaccinated group and once in 3 months for the controls.

Cell preparations

Various cell populations were prepared from mouse spleen according to the methods described previously with slight modifications.^{13,14} Briefly, lymphocytes containing T and B cells were obtained by passing single-cell suspensions of spleen through Sephadex G-10 columns. B cells were isolated from T/B cells by depleting T cells with anti-Thy-1.2, anti-Lyt-1.2 and C. T cells were enriched by applying T/B cells to a T-cell recovery kit (Biotex, Lab. Inc. Alberta, Canada).

DC and DC-enriched APC were isolated according to the methods of Crowley *et al.* with some modifications.¹⁶ In short, mouse spleens were injected with 1.0 ml collagenase (100 U/ml, *Clostridium histolyticum*, type III, Sigma Chemical Co., St. Louis, MO), cut into pieces, and incubated at 37° for 30 min in 1.0 ml collagenases (400 U/ml). A single-cell suspension of spleen was obtained by mechanical agitation with culture medium supplemented with 10% fetal calf serum. Single-cell suspensions from mice spleen were centrifuged on dense albumin column ($P=1.082$) at 4° for 30 min at 10 000 g, and the cells at the interface were collected and cultured for 90 min at 37° on plastic surface. Non-adherent cells were discarded and the adherent cells were cultured for an additional 18 hr at 37° after which it became non-adherent. The non-adherent cells after an 18-hr culture were recovered and depleted of contaminating T and B cells using anti-Thy-1.2, anti-Lyt-1.2, anti-CD45R plus C and were used as DC-enriched APC. In order to isolate the pure populations of DC, 18-hr non-adherent cells were depleted of macrophages by two additional adherence steps at 37° and were depleted of Fc-receptor-bearing cells by resetting with antibody-coated sheep erythrocytes. Fc-receptor-negative cells were treated with a mixture of anti-Thy-1.2, anti-Lyt-1.2, anti-CD45R plus C to eliminate contaminating T and B cells and were used as pure populations of DC.

Antigen-specific T lymphoblasts were prepared according to the method of Inaba *et al.*¹⁷ with some modifications by culturing T cells with HBsAg in culture medium supplemented with mouse serum for 10 days, after which the viable cells were collected by applying on a density gradient, Lympholyte-M.

Cell culture for proliferation and antibody production

This was done according to the already described methods with slight modifications.^{13,14} Proliferation of T cells in response to concanavalin A (Con A) (2.0 μ g/ml) and that B cells to lipopolysaccharides (LPS) (50.0 μ g/ml) were assayed by measuring [³H]thymidine ([³H]TdR) incorporation (1.0 μ Ci/ml) during the last 12 hr of culture period of 56 and 72 hr, respectively.

For non-specific immunoglobulin production, purified B cells at 3×10^5 cells/well were cultured with LPS (30 μ g/ml)

in 200 µl of medium in 96-well culture plates, and culture supernatants were collected 5 days later.

The stimulatory capacity of various doses of DC was determined by monitoring proliferation of allogenic T cells from C3H/He mice, sodium periodate (NaIO₄)-treated autologous T cells and HBsAg-specific T lymphoblasts. DNA synthesis was assayed by measuring the incorporation of [³H]TdR at a dose of 1.0 µCi/ml during the last 16 hr of a culture period of 48 hr (oxidative mitogenesis) and 120 hr (allogenic MLR and HBsAg-specific proliferation).

Cytokine productions in cultures

The capacity of DC to produce interleukin-12 (IL-12) was evaluated by culturing 2×10^5 DC/200 µl in 96-well culture plates for 48 hr. Production of IL-2 and tumour necrosis factor-α (TNF-α) were seen in supernatants from cultures containing T lymphocytes from HBsAg-injected normal C57BL/6 mice and DC from different sources.

Estimations of cytokines

IL-2, IL-12 and TNF-α were estimated in supernatants by ELISA methods using commercial kits (IL-2, IL-12 and TNF-α kit, BioSource International, CA). Samples were incubated on microtitre plates coated with respective monoclonal antibodies, followed by addition of a biotinylated second antibody. After removal of excess antibody, colour development was finished by enzymatic reaction of streptavidin peroxidase, the intensity of which was directly proportional to the concentrations of respective cytokines in the samples. The amounts of cytokines in the samples were estimated by calibrating the OD values of the samples with the OD values of the standards, supplied with the kits. The lowest levels of cytokines detectable by these kits were; IL-2, 15.0 pg/ml; IL-12, 7.8 pg/ml; TNF-α, 19.5 pg/ml.

Cytofluorometry

This was done according to a previously described procedure.^{13,14} Briefly, cells were stained with an optimum dilution of FITC-conjugated specific antibody directly or were stained with an optimum dilution of primary antibody followed by FITC-conjugated antibody, washed and suspended in PBS containing 1% bovine serum albumin and 0.02% sodium azide and finally FITC⁺ cells were counted in a flow cytometer (EPICS profile, Coulter Corp, Hialeah, FL). Cells unstained and stained with FITC-conjugated second antibody alone served as a control.

Statistical analysis

Data were expressed as means ± SD. Statistical analyses were performed using Student's *t*-test, the Fisher Exact test and Mann-Whitney's *U*-test, as indicated. In all statistical comparisons, a *P*-value < 0.05 was used to indicate a significant difference.

RESULTS

Heterogeneous outcome of vaccine therapy in HBV-Tg

Those HBV-Tg that became completely negative for both HBsAg and HBeAg in sera and reduced HBV DNA due to 12 months of vaccine therapy were regarded as vaccine responders. The placebo-recipients and the control HBV-Tg

did not show significant alterations in the titres of HBsAg and HBeAg after the end of 12 months of vaccine therapy. Although, all 35 vaccine-recipient HBV-Tg were of the same age and sex and had similar titres to HBV-related markers, 22 of the 35 vaccine-recipient HBV-Tg became completely negative for HBsAg and HBeAg in sera (vaccine responders), whereas, HBsAg and HBeAg remained mostly unchanged in 10 vaccine-recipient HBV-Tg (vaccine non-responders) (Table 1). Serum HBV DNA were reduced in all vaccine responders but not in any vaccine non-responders (data not shown). In 20 vaccine responders, circulating anti-HBs were detected in sera.

Heterogeneity of DC function in HBV-Tg

As HBV-markers had no prognostic importance during vaccine therapy in HBV-Tg, host-derived factors, such as function of lymphocytes and APC were analysed in untreated HBV-Tg. The proliferation of T cells from HBV-Tg in response to Con A in the presence of APC from normal C57BL/6 mice (ranged between 23 564 c.p.m. and 29 219 c.p.m., *n* = 12), B cells to LPS (ranged between 32 456 c.p.m. and 37 213 c.p.m., *n* = 12), the production of polyclonal IgM (ranged between 5.4 µg and 5.8 µg, *n* = 12) and IgG in response to LPS (ranged between 5.3 µg and 5.8 µg, *n* = 12) were almost similar among different HBV-Tg.

But, the levels of blastogenesis in allogenic MLR-containing T cells from C3H/He mice and pure populations of DC (*n* = 9) or DC-enriched APC (*n* = 13) from HBV-Tg were

Table 1. Outcome of vaccine therapy in HBV-transgenic mice*

	HBsAg	HBeAg	Anti-HBs
<i>Vaccine-recipients HBV-Tg</i>			
Before	2 ⁷ † (<i>n</i> = 35)‡	2 ⁴ (<i>n</i> = 35)	2 ⁰ (<i>n</i> = 35)
After			
Responders	2 ⁰ (<i>n</i> = 22)	2 ⁰ (<i>n</i> = 22)	2 ⁷ (<i>n</i> = 10) 2 ⁵ (<i>n</i> = 8) 2 ⁴ (<i>n</i> = 2) 2 ² (<i>n</i> = 2)
Semi-responders	2 ⁴ (<i>n</i> = 3)	2 ³ (<i>n</i> = 3)	2 ⁰ (<i>n</i> = 3)
Non-responders	2 ⁶ (<i>n</i> = 6) 2 ⁵ (<i>n</i> = 4)	2 ⁴ (<i>n</i> = 8) 2 ³ (<i>n</i> = 2)	2 ⁰ (<i>n</i> = 10)
<i>Placebo-recipients HBV-Tg</i>			
Before	2 ⁷ (<i>n</i> = 15)	2 ⁴ (<i>n</i> = 15)	2 ⁰ (<i>n</i> = 15)
After	2 ⁷ (<i>n</i> = 12) 2 ⁶ (<i>n</i> = 3)	2 ⁴ (<i>n</i> = 14) 2 ³ (<i>n</i> = 1)	2 ⁰ (<i>n</i> = 15)
<i>Control HBV-Tg</i>			
Before	2 ⁷ (<i>n</i> = 10)	2 ⁴ (<i>n</i> = 10)	2 ⁰ (<i>n</i> = 10)
After	2 ⁷ (<i>n</i> = 7) 2 ⁶ (<i>n</i> = 3)	2 ⁴ (<i>n</i> = 8) 2 ³ (<i>n</i> = 2)	2 ⁰ (<i>n</i> = 10)

*Three groups of HBV-Tg having similar levels of HBV-markers in sera were injected with HBsAg in CFA (Vaccine-recipients) or CFA only (Placebo-recipients) once a month for 12 consecutive months or were left untreated (Controls), and the response to vaccine therapy was evaluated 12 months after the start of therapy. † Titres of HBsAg, HBeAg and anti-HBs were shown by haemagglutination; levels > 2² indicated a positive response and the end-point dilution was reached by a twofold dilution. ‡ indicated number of HBV-Tg.

extremely heterogeneous (Table 2). The levels of blastogenesis ranged between 14 234 c.p.m. and 42 387 c.p.m. when pure populations of DC were used as stimulators and were between 8324 c.p.m. and 31 254 c.p.m. when DC-enriched APC were used as stimulators. The heterogeneous levels of blastogenesis in allogenic MLR could not be attributable to the T-cell populations because all T cells were taken from a pooled source of T cells from normal C3H/He mice. Moreover, when the same T cells from C3H/He mice were cultured with DC from normal C57BL/6 mice in allogenic MLR, the levels of blastogenesis were mostly similar (38 679 c.p.m. to 46 654 c.p.m.). These data showed the heterogeneity in the stimulatory capacity of DC among HBV-Tg, although all HBV-Tg were of same age, sex and MHC background and had similar levels of HBV-markers.

Heterogeneity in the serum titres of anti-KLH IgG in HBV-Tg

Thirty-three HBV-Tg having similar levels of HBsAg, HBeAg and HBV DNA and 18 normal C57BL/6 mice were injected twice with 5 µg of KLH in PBS (a dose known as an optimum dose to induce anti-KLH IgG in all normal C57BL/6 mice¹³), i.p., at an interval of 2 weeks and the titres of anti-KLH IgG were estimated 6 weeks after first injection. All normal mice produced anti-KLH IgG in sera, as expected. But, the response of HBV-Tg to KLH was heterogeneous in HBV-Tg. Nineteen of the 33 HBV-Tg produced significant levels of anti-KLH IgG (>10.0 DTgU/µl) (KLH-responders) in sera, whereas, 10 HBV-Tg could not produce any anti-KLH IgG (KLH-non responders) and four HBV-Tg produced only very small amounts of anti-KLH IgG (1.0 – 2.0 DTgU/µl).

Table 2. Heterogeneity of stimulatory capacity of DC from HBV-Tg in allogenic MLR

Range of c.p.m.	No. of HBV-Tg	c.p.m.
<i>Pure DC populations</i>		
10 000–20 000	3	14 234, 16 354, 17 453
20 000–30 000	2	28 767, 29 876
30 000–40 000	3	32 767, 34 357,
>40 000	1	42 387
<i>DC-enriched antigen-presenting cells</i>		
5000–10 000	2	8324, 9245
10 000–20 000	5	13 265, 14 356, 16 254, 17 564, 19 238
20 000–30 000	5	23 654, 27 654, 28 546, 28 798, 29 123
>30 000	1	31 254

Pure populations of DC ($>90\%$ DC were positive for N418, a DC-specific marker and expressed very high levels of MHC class II) and DC-enriched APC (containing 35–65% N418 positive cells) were isolated from the spleen of HBV-Tg with same age and sex and expressing similar levels of HBsAg, HBeAg and HBV DNA, as described. T cells were isolated from normal C3H/He mice (H-2^b) using a T-cell recovery kit and were cultured with mitomycin-C-treated DC populations from HBV-Tg in allogenic MLR for 5 days. The incorporation of [³H]TdR during the last 16 hr of the total culture period of 5 days was expressed as c.p.m. There was marked heterogeneity in the levels of blastogenesis in allogenic MLR among HBV-Tg.

Relationship between stimulatory capacity of DC and levels of serum anti-KLH in HBV-Tg

We found marked heterogeneity, both in the stimulatory capacity of DC and in the levels of anti-KLH IgG in HBV-Tg, but we were not sure whether these two independent observations were related or not because the experiments regarding the stimulatory capacity of DC and production of anti-KLH IgG were carried out in different HBV-Tg. In order to have further insights in this regard, 55 HBV-Tg with similar levels of HBV-markers were injected with 5 µg of KLH in PBS, i.p., and were boosted with the same dose after 2 weeks. The levels of anti-KLH IgG were estimated in sera 6 weeks after the first injection. Out of 55 HBV-Tg, 34 produced high levels of anti-KLH IgG (>10.0 DTgU/µl) (KLH-responders) in sera, whereas, 16 HBV-Tg could not produce any anti-KLH IgG (KLH-non responders). All HBV-Tg were then killed and T and B lymphocytes and DC-enriched APC were isolated from the spleen. Table 3 shows that the proliferative capacity of T cells in response to Con A ($34\,254 \pm 6576$ c.p.m. versus $37\,243 \pm 6876$ c.p.m., $P > 0.1$), of B cells to LPS ($27\,267 \pm 4532$ c.p.m. versus $25\,467 \pm 6502$ c.p.m., $P < 0.1$), the production of polyclonal IgM (7.2 ± 1.1 µg/µl versus 6.8 ± 1.2 µg/µl) and IgG (6.8 ± 1.7 µg/µl versus 6.5 ± 1.5 µg/µl) by B cells in response to LPS were not different between KLH-responders

Table 3. Increased stimulatory capacity of DC from KLH-responders HBV-Tg

	KLH responders	KLH non-responders
<i>Lymphocyte function</i>		
Lymphocyte proliferation	(c.p.m.)	(c.p.m.)
Con A-induced	$34\,254 \pm 6576$	$37\,243 \pm 6876$
LPS-induced	$27\,267 \pm 4532$	$25\,467 \pm 6502$
<i>Production of immunoglobulins (µg/ml)</i>		
IgM	7.2 ± 1.1	6.8 ± 1.2
IgG	6.8 ± 1.7	6.5 ± 1.5
<i>DC function</i>		
Allogenic MLR	$41\,254 \pm 9532^*$	$17\,412 \pm 5439$
Oxidative mitogenesis	$37\,239 \pm 6723^*$	$14\,267 \pm 6120$

Injection of 55 HBV-Tg with same age and sex and HBV-markers with two injections with KLH in PBS showed 34 HBV-Tg produced >10 DTgU/µl of anti-KLH IgG (KLH-responders), whereas 16 HBV-Tg produced no anti-KLH IgG (KLH-non responders) in sera 6 weeks after the first injection with KLH. The proliferative response of T cells to Con A (in presence of APC from normal C57BL/6 mice) and of B cells to LPS were assayed by measuring the incorporation of [³H]TdR. Production of immunoglobulins by B lymphocytes in response to LPS was estimated by measuring the IgM and IgG in culture supernatants. The lymphocyte function was shown as mean \pm SD of duplicate assays of triplicate cultures of seven separate experiments. Stimulatory capacity of DC-enriched APC was estimated from the levels of blastogenesis in allogenic MLR and oxidative mitogenesis, as described in the Materials and Methods. Data were shown as c.p.m. of mean \pm SD of duplicate assays of triplicate cultures of 12 separate experiments for KLH-responders and eight separate experiments for KLH-non-responders. * $P < 0.05$ compared with KLH-non-responders.

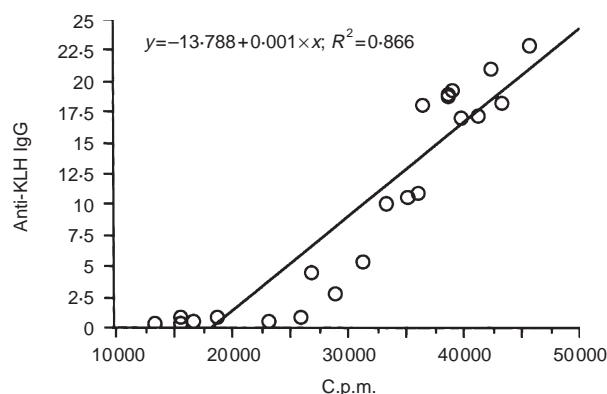


Figure 1. Correlation of anti-KLH IgG titres (DTgU) and stimulatory capacity of DC (counts per minutes [c.p.m.]) in HBV-Tg. HBV-Tg were injected with 5 µg of KLH in PBS, i.p., twice at an interval of 2 weeks and anti-KLH IgG titres were measured in sera 4 weeks after second immunization. Allogenic MLR was done using DC-enriched APC from HBV-Tg and T cells from C3H/He mice. The incorporation of [³H]TdR (c.p.m.) during the last 16 hr of the 5-day culture period indicated the stimulatory capacity of DC. The stimulatory capacity of DC (c.p.m.) positively correlated with the anti-KLH IgG titres ($r = 0.866$, $P < 0.0001$) in HBV-Tg.

and KLH-non-responders. But, the stimulatory capacity of DC-enriched APC from KLH-responders was significantly higher compared with the stimulatory capacity of DC-enriched APC from KLH-non-responders in both allogenic MLR and oxidative mitogenesis (Table 3).

When the stimulatory capacity of DC-enriched APC in allogenic MLR (blastogenesis in c.p.m.) and the anti-KLH IgG titres in sera (DTgU/µl) from the same HBV-Tg were plotted ($n = 23$), a good correlation was seen between these two immunological markers and we used the anti-KLH IgG titres as an indicator of DC function in HBV-Tg ($r = 0.866$, $P < 0.0001$, Fig. 1).

Relationship between function of DC and the outcome of vaccine therapy

The experiments described in this series showed that the stimulatory capacity of DC could be predicted 6 weeks after injecting KLH by estimating anti-KLH IgG in sera. In order to evaluate the utility of titres of anti-KLH IgG as a marker of DC function and also to assess the role of DC, if any, as a prognostic marker during vaccine therapy, two trials of vaccine therapy were conducted in HBV-Tg. In the first one, a group of 17 HBV-Tg with similar levels of HBsAg, HBeAg and HBV DNA were injected with vaccine containing 10 µg of HBsAg in CFA and 5 µg of KLH at the same time. KLH was injected once more after 2 weeks. All HBV-Tg were bled from the tail vein 4 weeks after the booster injection and the titres of anti-KLH IgG were estimated in sera. Eight of the 17 HBV-Tg produced more than 10.0 DTgU/µl of anti-KLH IgG (high-responders), whereas three did not produce any anti-KLH IgG (non-responders) and six HBV-Tg showed very small amounts of anti-KLH IgG (0–3.0 DTgU/µl) in sera. All 17 HBV-Tg were assigned to receive monthly injections of vaccine containing HBsAg in CFA for 12 consecutive months.

As shown in Table 4, all eight HBV-Tg that were high-

Table 4. Relationship between the titres of anti-KLH IgG and response to vaccine therapy

HBV-Tg* No.	anti-KLH IgG† (> 1.0 DTgU/µl)		HBsAg ($\geq 2^2$)		HBeAg ($\geq 2^2$)		anti-HBs ($\geq 2^2$)	
	B	A	B	A	B	A	B	A
1	0.3	10.8	2 ⁷	2 ¹	2 ⁴	2 ¹	2 ⁰	2 ⁵
2	0.2	12.3	2 ⁷	2 ¹	2 ⁴	2 ¹	2 ⁰	2 ⁵
3	0.3	13.4	2 ⁷	2 ¹	2 ⁴	2 ¹	2 ⁰	2 ⁴
4	0.3	19.5	2 ⁷	2 ¹	2 ⁴	2 ¹	2 ⁰	2 ³
5	0.4	14.8	2 ⁷	2 ¹	2 ⁴	2 ¹	2 ⁰	2 ⁴
6	0.4	15.2	2 ⁷	2 ¹	2 ⁴	2 ¹	2 ⁰	2 ⁵
7	0.3	13.3	2 ⁷	2 ¹	2 ⁴	2 ¹	2 ⁰	2 ⁴
8	0.4	10.7	2 ⁷	2 ¹	2 ⁴	2 ¹	2 ⁰	2 ⁴
9	0.5	2.9	2 ⁷	2 ⁷	2 ⁴	2 ⁴	2 ⁰	2 ⁰
10	0.4	1.8	2 ⁷	2 ⁶	2 ⁴	2 ³	2 ⁰	2 ⁰
11	0.4	2.6	2 ⁷	2 ⁷	2 ⁴	2 ⁴	2 ⁰	2 ¹
12	0.5	2.1	2 ⁷	2 ⁵	2 ⁴	2 ⁴	2 ⁰	2 ¹
13	0.3	2.4	2 ⁷	2 ⁶	2 ⁴	2 ³	2 ⁰	2 ¹
14	0.4	1.9	2 ⁷	2 ⁶	2 ⁴	2 ⁴	2 ⁰	2 ¹
15	0.3	0.7	2 ⁷	2 ⁵	2 ⁴	2 ⁴	2 ⁰	2 ¹
16	0.4	0.4	2 ⁷	2 ⁷	2 ⁴	2 ³	2 ⁰	2 ⁰
17	0.4	0.5	2 ⁷	2 ⁷	2 ⁴	2 ⁴	2 ⁰	2 ¹

*Seventeen HBV-Tg with HBsAg and HBeAg titres of 2⁷ and 2⁴, respectively, and with no anti-KLH IgG in sera were injected with 5 µg of KLH in PBS and vaccine containing 10 µg of HBsAg in CFA at the same time. †KLH (≥ 1.0 DTgU/µl) was injected once more after 2 weeks and anti-KLH IgG in sera was estimated 6 weeks after the first injection with KLH; the anti-KLH IgG levels > 1.0 DTgU/µl indicated a positive anti-KLH IgG response. Vaccination with HBsAg in CFA was continued once a month for 12 consecutive months. B, before the start of vaccine therapy and KLH injection. A, after 6 weeks in the case of anti-KLH IgG titres, and after 12 months in the case of HBV-markers. HBsAg, HBeAg, and anti-HBs in sera were screened by haemagglutination and end-point titration was done by twofold serial dilution and levels $> 2^2$ indicated a positive response. Mice nos 1–8 were KLH-vaccine responders, whereas, mice nos 9–17 were KLH-non responders. All KLH-responders also became vaccine responders.

responders to KLH became completely negative for HBsAg, HBeAg and developed anti-HBs in sera after 12 months of vaccine therapy. These vaccine-responding HBV-Tg also reduced HBV DNA in sera compared with the prevaccinated levels (Fig. 2). Moreover, *in situ* hybridization of liver also showed significantly reduced levels of signals for HBV DNA in vaccine-responding HBV-Tg compared with their prevaccinated levels and vaccine non-responders (Fig. 3). On the other hand, nine HBV-Tg that showed either no or very little anti-KLH IgG in sera 6 weeks after injecting KLH became vaccine non-responders and HBV-markers remained mostly unchanged in these HBV-Tg (Table 4).

Although, these experiments showed a direct relationship between the anti-KLH IgG titres and response to vaccine therapy but as KLH and HBsAg were injected together, it might be possible that injection with KLH might have modulated the outcome of vaccine therapy in responders. Again, it was possible that injection with HBsAg in CFA influenced the titres of anti-KLH IgG, when both KLH and HBsAg were injected together. This issue was addressed by conducting a second trial of vaccine therapy in 26 HBV-Tg in which all

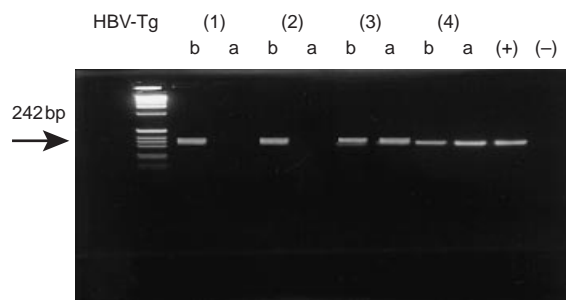


Figure 2. Reduced levels of HBV DNA in sera from vaccine responders. Total DNA from sera of vaccine responder and non-responder HBV-Tg were isolated, measured and adjusted to different concentrations. Total serum DNA (0.5 ng) in two representative cases from vaccine responders (HBV-Tg No. 1 and 2) and two representative cases from vaccine non-responders (HBV-Tg no. 3 and 4) before the start of vaccine therapy (b) and after the 12 months of therapy (a) were amplified by PCR and allowed to run on agarose gel. A band 242 bp in length indicated the presence of HBV DNA. Lanes indicating (+), and (–) indicated the known positive control and negative control, respectively.

HBV-Tg were injected with 5 µg of KLH in PBS, twice at an interval of 2 weeks and anti-KLH IgG levels were seen 6 weeks after the first injection. All 26 HBV-Tg were given a rest for 8 weeks after a booster injection with KLH. Then the first

dose of vaccine therapy was started and monthly injection with vaccine was continued for 12 months. Sixteen HBV-Tg showed high titres of anti-KLH IgG (>10.0 DTgU/µl) and 15 of these became complete vaccine responders, while one that became negative for HBsAg was slightly positive for HBeAg (HBeAg titre 2^3 by reversed passive haemagglutination method). On the other hand, all 10 HBV-Tg that were either low or no responders to KLH (anti-KLH IgG, 0–3.0 DTgU/µl) were vaccine non-responders.

Selective activation of DC following vaccination

Data from our experiments showed that HBV-Tg with potent DC function had better stimulatory capacity in allogenic MLR, produced high titres of anti-KLH IgG in sera 6 weeks after injecting KLH and ultimately responded to vaccine therapy, which was evaluated 12 months after the start of therapy. HBV-Tg with poor DC function showed no such activity. This indicated that vaccination with HBsAg in HBV-Tg might cause different types of cellular events depending on whether HBV-Tg had a potent DC function or poor DC function. To have more insights into this and to reconfirm the utility of anti-KLH IgG titres as a marker of DC function, 46 HBV-Tg having HBsAg and HBeAg titres of 2^7 and 2^4 in sera were injected twice with 5 µg of KLH in PBS. Twenty-one HBV-Tg produced anti-KLH IgG more than

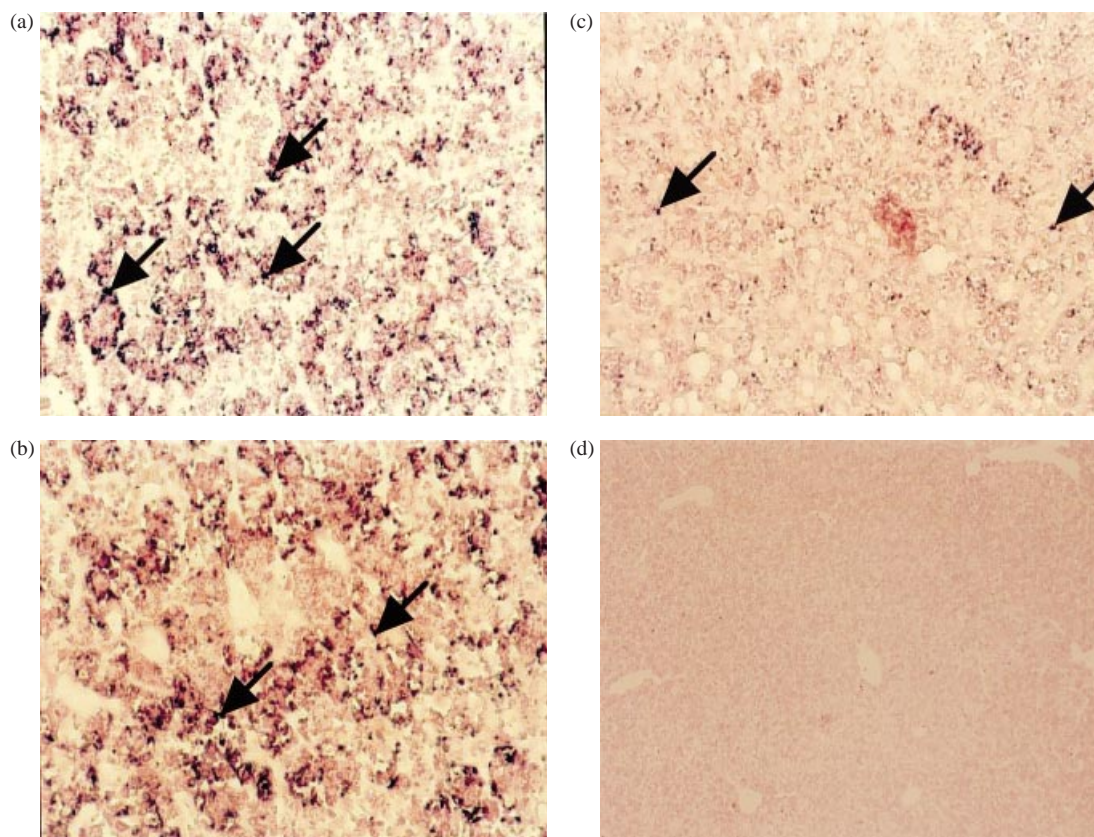


Figure 3. *In situ* hybridization for HBV DNA in liver from HBV-Tg before vaccine therapy (a), after vaccine therapy in vaccine responders (b) and in vaccine non-responders (c) and in liver from normal C57BL/6 mice (d). HBV DNA were seen as black granules in the liver using the digoxigenin-labelled HBV DNA probe. A decrease in the signals for HBV DNA was found in vaccine responders HBV-Tg compared with HBV-Tg before vaccine therapy and in vaccine non-responder HBV-Tg after vaccine therapy. In control liver (d), there was no signal for HBV DNA.

Table 5. HBsAg-induced up-regulation of MHC class II and CD86 on DC from KLH-high responders

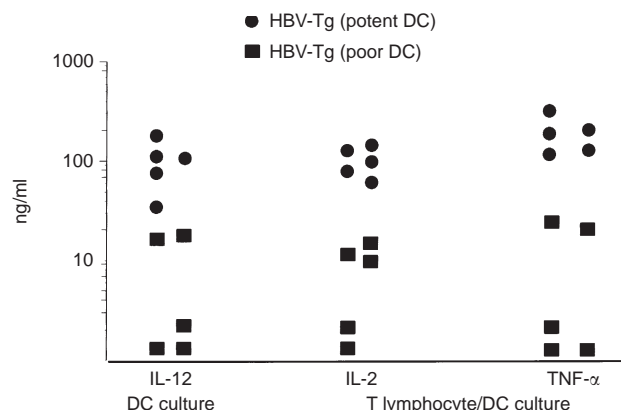
		KLH-high-responders	KLH-non responders
<i>MHC class II</i>			
PBS		223.0 ± 17.0	198.0 ± 15.0
HBsAg	in	478.0 ± 30.0*	214.0 ± 22.0
CFA			
<i>CD86</i>			
PBS		173.0 ± 7.0	170.0 ± 13.0
HBsAg	in	355.0 ± 23.0*	167.0 ± 21.0
CFA			
<i>CD80</i>			
PBS		154.0 ± 19.0	132.0 ± 29.0
HBsAg	in	189.0 ± 34.0	145.0 ± 35.0
CFA			

HBV-Tg with similar levels of HBsAg, and HBeAg were injected with 5 µg of KLH in PBS, i.p. at an interval of 2 weeks and anti-KLH antibody was estimated in sera 6 weeks after the first injection and expressed in DTU/g/µl. HBV-Tg producing >10.0 DTgU/µl of anti-KLH antibody were regarded as KLH-high-responders, whereas, those that could not produce anti-KLH antibody were regarded as KLH-non-responders. All HBV-Tg were kept on a normal diet for 5 months. Half of the HBV-Tg from both KLH-high-responders and non-responders groups were injected with 10 µg of HBsAg in CFA and the other half were injected with PBS. DC were isolated from all HBV-Tg, 3 days after injecting HBsAg in CFA or PBS. The expression of MHC class II, CD86, and CD80 was analysed by direct flow cytometry and data were shown as mean ± SD of fluorescence intensities ($n=3$). Injection of HBsAg in CFA caused up-regulation of MHC class II and CD86 on DC from KLH-high-responder HBV-Tg but not in KLH-non-responder HBV-Tg; * $P \leq 0.05$.

10.0 DTgU/µl (KLH-high-responders) and 12 HBV-Tg could not produce any anti-KLH IgG in sera 4 weeks after booster injection with KLH (KLH-non-responders). All HBV-Tg were rested and maintained on a normal diet for 5 months. Then these HBV-Tg were injected with either 10 µg of HBsAg in CFA or PBS and pure populations of DC were isolated from spleen 3 days after injections. As shown in Table 5, the expression of MHC class II and CD86 on DC was significantly higher in KLH-responders HBV-Tg compared with KLH-non-responders HBV-Tg following injection with HBsAg in CFA, although the expression of these antigens was not significantly different between KLH-high-responder and KLH-non-responder HBV-Tg following injection with PBS. The levels of IL-12, IL-2 and TNF-α were also significantly higher in cultures containing DC from KLH-high-responders compared with cultures containing DC from KLH-non-responders (Fig. 4). Although significant amounts of IL-12, IL-2 and TNF-α were produced in all five cultures containing DC from KLH-high-responder HBV-Tg (HBV-Tg with potent DC function), cultures containing DC from KLH-non-responder HBV-Tg (HBV-Tg with poor DC function) did not show any detectable levels of IL-12 in three, IL-2 in two and TNF-α in three cultures.

Induction of HBsAg-specific proliferation by DC from KLH-responders

DC, isolated from KLH-high-responders and KLH-non-responders were pulsed with HBsAg, as described in the

**Figure 4.** Production of IL-12 by dendritic cells and IL-2 and TNF-α in culture containing T cells from DC from HBV-Tg with potent DC function and HBV-Tg with poor DC function. Dendritic cells (2×10^5 cells/200 µl) from HBV-Tg with potent DC function (●) and from HBV-Tg with poor DC function (■) were cultured for 48 hr with HBsAg and IL-12 in culture supernatants were measured by an ELISA. The production of IL-2 and TNF-α was estimated in supernatants from cultures containing T cells from HBsAg-primed normal mice and dendritic cells from HBV-Tg with potent DC function (●) and from HBV-Tg with poor DC function (■).**Table 6.** Efficient induction of HBsAg-specific proliferation by DC from KLH-high-responders

Unpulsed dendritic cells	HBsAg-pulsed dendritic cells	Stimulation index (positivity ≥ 2.0)
KLH-high-responders		7.3 ± 1.1
	KLH-high-responders	14.3 ± 2.1
KLH-non-responders		1.2 ± 0.3
	KLH-non-responders	1.3 ± 0.2

Normal C57BL/6 were injected with HBsAg (10 µg/mouse) in CFA, twice at an interval of 4 weeks and killed 2 weeks after the last immunization and T cells were isolated from spleen. HBsAg-specific T lymphoblasts were generated from splenic T cells by culturing with HBsAg for 10 days. The proliferation of HBsAg-specific lymphoblasts (3×10^5) in response to DC or HBsAg-pulsed DC (1.5×10^4) without or with HBsAg (10 ng/ml) HBsAg in culture was estimated from the incorporation of [3 H]TdR (expressed as c.p.m.) during the last 16 hr of a total culture period of 5 days. Stimulation index was calculated by dividing the levels of c.p.m. in cultures containing HBsAg with c.p.m. in cultures without HBsAg. A stimulation index of >2.0 indicated a significant proliferation. Data were shown as mean ± SD of duplicate assays of triplicate cultures of four separate experiments.

Materials and Methods. Unpulsed DC and HBsAg-pulsed DC were used to induce proliferation of HBsAg-specific T lymphoblasts, isolated from normal C57BL/6 mice injected twice with HBsAg in CFA. Unpulsed DC and HBsAg-pulsed DC from KLH-high-responders but not from KLH-non-responders induced significant proliferation of HBsAg-specific T lymphoblasts (Table 6).

DISCUSSION

We and others have shown that HBV-Tg represents a useful animal model of HBV-carrier state to elucidate the host-virus interactions, when they are used to address questions that

cannot be otherwise approached by the existing methodology in human HBV-carriers.^{11–14} A placebo-controlled, double blind trial of vaccine therapy in 35 HBV-Tg with same age, sex and MHC background, and with similar levels of HBV-markers (HBsAg, HBeAg and HBV DNA) showed that 22 HBV-Tg became complete responders and became negative for HBsAg, HBeAg and reduced HBV DNA, whereas, HBV-markers remained unchanged in 10 HBV-Tg (Table 1). The outcome of the vaccine therapy was similar to what we and others have reported about the heterogeneity of outcome of vaccine therapy in human and murine HBV-carriers. Moreover, it became apparent that the prevaccinated titres of HBV-related markers have a limited role, if any, to influence the final outcome of this immune therapy.

The proliferation and production of immunoglobulins in response to polyclonal mitogens by lymphocytes were mostly similar among untreated mice, vaccine responders and vaccine non-responders (Results and Table 3); the direct role of lymphocytes to influence the outcome of vaccine therapy could not be substantiated.

As the outcome of vaccine therapy in HBV-Tg was related neither to the titres of HBV-markers nor to the function of lymphocytes, the function of DC, the most potent APC, was analysed to gain insight into the prognostic marker of vaccine therapy. A series of experiments revealed that the stimulatory capacity of both pure populations of DC and DC-enriched APC was extremely heterogeneous among untreated HBV-Tg, although all were of same age, sex and MHC background and all had similar levels of HBsAg and HBeAg in sera (Table 2). The data from these experiments led us to postulate that the stimulatory capacity of DC prior to vaccination might have influenced the outcome of vaccine therapy. Although this could be tested by checking the function of DC prior to vaccination and by analysing the outcome of vaccine therapy, it was technically impossible to evaluate both in the same HBV-Tg because mice must be killed to isolate the DC. In order to solve this, we looked for an immunological marker capable of reflecting the function of DC in HBV-Tg. We failed to establish any direct relationship between the stimulatory capacity of DC in allogenic MLR and serum levels of cytokines, such as IL-12, IL-2 and TNF- α (data not shown) in HBV-Tg. But, a very good correlation was found between the serum titres of anti-KLH IgG and the stimulatory capacity of DC in allogenic MLR ($r=0.866$, $P<0.0001$); this allowed us to evaluate both the function of DC (from the anti-KLH IgG titres in sera) at the beginning of the vaccine therapy and the outcome of vaccine therapy 12 months after the start of therapy in the same HBV-Tg (Fig. 1).

A fresh trial of vaccine therapy was undertaken in 17 HBV-Tg with similar levels of HBsAg, HBeAg and HBV DNA. The serum anti-KLH IgG levels 6 weeks after the start of vaccine therapy showed that eight and nine HBV-Tg had potent and poor DC function, respectively. Vaccine therapy was continued for 12 months and the outcome was evaluated in all 17 HBV-Tg. All eight HBV-Tg, that had potent DC function were vaccine responders and became negative for HBsAg, HBeAg, reduced HBV DNA in sera (Fig. 2) and liver (Fig. 3) and developed anti-HBs in sera (Table 4). On the other hand, all nine HBV-Tg with poor DC function became vaccine non-responders and there were no significant changes in HBV-markers in any of these HBV-Tg (Table 4).

A second trial of vaccine therapy in 26 HBV-Tg in which the first dose of vaccine therapy was started 2 months after injecting booster dose of KLH reconfirmed the relationship between the DC function before vaccine therapy and the outcome of vaccine therapy (see the Results).

The importance of DC during an immune response and defective function of APC including DC in the pathogenesis of malignancies^{18–22} and chronic infections^{13,14} have already been shown. Vaccine therapy in which HBsAg have been coupled to anti-HBs, solid matrix and lipopeptide to ensure better antigen presentation has shown better therapeutic potential.^{23,24} DC-pulsed with tumour antigen have shown anti-tumour activities. Thus, there are increasing arguments in favour of a critical role of DC in immune therapy but this is the first report which has shown the prognostic importance of a host-derived factor like DC during an immune therapy like vaccine therapy. Recently, Hsu *et al.*²¹ have reported that vaccination of four patients with B lymphoma using autologous antigen-pulsed DC resulted in heterogeneous outcome. Although, there might be multiple factors for this heterogeneity of response in lymphoma patients, but if the DC function prior to therapy was known in all four cases, then we might have a better understanding about the prognostic importance of DC during tumour immune therapy.

Although, we have shown the critical role of the stimulatory capacity of DC as a prognostic marker during vaccine therapy in HBV-Tg, these findings would have very limited practical implications in human HBV-carriers. The levels of blastogenesis in allogenic MLR containing T cells from C3H/He mice and DC from HBV-Tg represented the stimulatory capacity of DC in HBV-Tg because all T-cell populations were taken from an inbred strain of normal mice (C3H/He mice), which had similar proliferative capacity. But in human the levels of blastogenesis in allogenic MLR are dependent on the stimulatory capacity of DC as well as on the proliferative capacity of T cells and both of these cell populations show marked variations in their respective functions even among normal individuals.

In order to address this and to have a clinical implication of our observations in murine HBV-carriers, another series of experiments were conducted in which the cellular events following vaccination in HBV-Tg with both potent and poor DC function were studied. The levels of MHC class II and CD86 antigens on DC from HBV-Tg with both potent and poor function of DC were almost similar *in situ* (data not shown) and when these were injected with PBS (Table 5). But, the injection of HBsAg in CFA induced completely different types of stimulation between HBV-Tg with potent and poor DC function. When HBV-Tg with potent DC function were injected with HBsAg in CFA, the expressions of MHC class II and CD86 antigens on DC were significantly up-regulated (Table 5). Similarly, increased production of IL-12, IL-2 and TNF- α were seen in cultures containing DC from HBV-Tg with potent DC function (Fig. 4). The up-regulation of surface antigens and increased production of cytokines were not seen when HBV-Tg with poor DC function were injected with HBsAg in CFA. These data indicated that DC from HBV-Tg with potent DC function had the capability to be activated following injection with vaccine containing HBsAg and this is probably the vital events leading to therapeutic activity of vaccine therapy. This was further confirmed by showing that

DC from HBV-Tg with potent DC function was able to induce HBsAg-specific proliferation of HBsAg-specific T lymphoblasts (Table 6). We have already shown the role of activation of DC for anti-HBs production in HBV-Tg *in vitro*.²⁵ This observation showing the relationship between the activation of DC and the therapeutic potentiality of vaccine therapy is in line with what Matzinger²⁶ and Ridge *et al.*²⁷ have suggested regarding the activation of DC and initiation of an immune response. They have proposed that the primary distinction for immune response is made between dangerous and harmless entities by APC, which are activated to up-regulate costimulatory molecules only when induced by alarm signals from their environment.

The HBV-Tg used in these experiments were prepared on the genetic background of C57BL/6 mice, which are known as intermediate responders to HBsAg.²⁸ Further experiments using HBV-Tg prepared on different genetic backgrounds, such as HBsAg-high-responder mice (H-2^a haplotype)²⁸ or HBsAg-low-responder mice (H-2^{s-f})²⁸ might provide further insights in this regard.

Data from our experiments led us to postulate that human HBV-carriers, in whom injection with vaccine containing HBsAg would cause up-regulation of surface antigen like HLA DR and CD86 on DC would respond to vaccine therapy, although further experiments in clinical set up in human HBV-carriers would contribute more in this regard and experiments about DC activation in human HBV-carriers following vaccination with HBsAg are in progress in our laboratory.

We have not evaluated the role of DC as prognostic markers in other pathological conditions, in which immune therapy, especially DC-based immune therapy, has shown therapeutic potential but data from our experiments indicated that if the basal level of DC function can be improved *in situ*, then it is strongly predicted that the immune therapy would cause better activation of DC and would initiate a series of cellular events resulting in therapeutic activity of that particular therapy. Flt3 has been reported to increase the number and efficacy of DC *in vivo*²⁰ and the data from our experiments showing a prognostic importance of DC provided a scientific and logical basis to up-regulate the function of DC prior to immune therapy.

ACKNOWLEDGMENTS

The authors are grateful to Prof. K-I. Yamamura (Department of Molecular Genetics and Embryology, Kumamoto University School of Medicine, Kumamoto, Japan) for providing us with the HBV-transgenic mice. We are extremely grateful to the Research Foundation for Microbial Diseases of Osaka University, Osaka, Japan for generously supplying us with vaccine containing HBsAg and recombinant HBsAg. The study was supported by a Grant-in-Aid for Scientific Research (C) (No. 10670481, 1998–99) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

- DUSHEIKO G. & HOOFNAGLE J.H. (1991) Hepatitis B. *Oxford Textbook of Clinical Hepatology*, p. 571. Oxford University Press, New York.
- PETERS M., VIERLING J., GRENSHWIN M.E., MILLICH D., CHISARI F.V. & HOOFNAGLE J.H. (1991) Immunology and the liver. *Hepatology* **13**, 977.
- THOMAS H.C. (1991). Pathogenesis of chronic HBV infection and mechanisms of action of anti viral compounds. In: *Viral Hepatitis and Liver Disease* (eds HOLLINGER F.B., LEMON S. & MAGOLIS H.S.), p. 612. Williams & Wilkins, Baltimore.
- PERIOLLO R.P. (1991). Treatment of chronic hepatitis B. In: *Viral Hepatitis and Liver Disease* (eds HOLLINGER F.B., LEMON S. & MAGOLIS H.S.), p. 616. Williams & Wilkins, Baltimore.
- PERIOLLO R.P. (1993). Interferon in the management of chronic hepatitis B. *Dig Dis Sci* **38**, 577.
- POL S. (1995) Immunotherapy of chronic hepatitis B by anti HBV vaccine. *Biomed Pharmacother* **49**, 105.
- POL S., DRISS F., MICHEL M., NALPES B., BERTHELOT P. & BRECHOT C. (1994) Specific vaccine therapy in chronic hepatitis B infection. *Lancet* **344**, 342.
- WEN Y.-M., WU X.-H., HU D.-C., ZHANG Q.-P. & GUO S.-Q. (1995) Hepatitis B vaccine and anti-HBs complex as approach for vaccine therapy. *Lancet* **345**, 1575.
- ARAKI K., MIYAZAKI J., HINO O. *et al.* (1989) Expression and replication of hepatitis B virus genome in transgenic mice. *Proc Natl Aca Sci, USA* **86**, 207.
- CHISARI F.V. (1995). Hepatitis B virus transgenic mice: insights into the virus and disease. *Hepatology* **22**, 1316.
- GUILHOT S., GUIDOTTI L.G. & CHISARI F.V. (1993) Interleukin-2 down regulates hepatitis B virus gene expression in transgenic mice by a post transcriptional mechanism. *J Virol* **67**, 7444.
- AKBAR S.M.F., KAJINO K., TANIMOTO T. *et al.* (1997) Placebo-controlled trial of vaccination with hepatitis B virus surface antigen in hepatitis B virus transgenic mice. *J Hepatol* **26**, 131.
- AKBAR S.M.F., ONJI M., INABA K., YAMAMURA K. & OHTA Y. (1993) Low responsiveness of hepatitis B virus-transgenic mice in antibody response to T-cell-dependent antigen: defect in antigen-presenting activity of dendritic cell. *Immunology* **78**, 468.
- AKBAR S.M.F., INABA K. & ONJI M. (1996) Upregulation of MHC class II antigen on dendritic cells from hepatitis B virus transgenic mice: abrogation of immune response defect to a T-cell-dependent antigen. *Immunology* **87**, 519.
- METLAY J.P., WITMER-PACK M.D., AGGER R., CROWLEY M.T., LAWLESS D. & STEINMAN R.M. (1990) The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J Exp Med* **171**, 1753.
- CROWLEY M., INABA K., WITMET-PACK M. & STEINMAN R.M. (1989) The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell Immunol* **118**, 108.
- INABA K., METLAY J.P., CROWLEY M.T. & STEINMAN R.M. (1990) Dendritic cells pulsed with protein antigens *in vitro* can prime antigen-specific, MHC-restricted T cells *in situ*. *J Exp Med* **172**, 631.
- GRABBE S., BEISSERT S., SCHWARZ T. & GRANSTEIN R.D. (1995) Dendritic cells as initiators of tumor immune response; a possible strategy for immunotherapy? *Immunol Today* **16**, 117.
- GABRILOVICH D.I., NADAF S., CORAK J., BERZOFKY J.A. & CARBONE D.P. (1996) Dendritic cells in antitumor immune responses. *Cell Immunol* **170**, 111.
- LYNCH D.H., ANDREASEN A., MARASKOVSKY E., WHITMORE J., MILLER R.E. & SCHOH J.C.L. (1997) Flt3 ligand induces tumor regression and antitumor immune response *in vivo*. *Nat Med* **3**, 625.
- HSU F.J., BENIKE C., FAGNONI F. *et al.* (1996) Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* **2**, 52.
- CELLUZZI C.M. & FALO L.D. JR (1998) Physical interaction between dendritic cells and tumor cells results in an immunogen that induces protective and therapeutic tumor rejection. *J Immunol* **160**, 3081.
- VITIELLO A., ISHIOKA G., GREY H.M. *et al.* (1995) Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV

- infection.1. Induction of a primary cytotoxic T lymphocyte response in humans. *J Clin Invest* **95**, 341.
24. WEN Y.-M., XIONG S.-D. & ZHANG W. (1994) Solid matrix-antibody-antigen complex can clear viraemia and antigenaemia in persistent duck hepatitis B virus infection. *J Gen Virol* **75**, 335.
25. KUROSE K., AKBAR S.M.F., YAMAMOTO K. & ONJI M. (1997) Production of antibody to hepatitis B surface antigen (anti-HBs) by murine hepatitis B virus carriers: neonatal tolerance versus antigen presentation by dendritic cells. *Immunology* **92**, 494.
26. MATZINGER P. (1994) Tolerance, danger and extended family. *Annu Rev Immunol* **12**, 991.
27. RIDGE J.P., FUCHS E.J. & MATZINGER P. (1996) Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* **271**, 1723.
28. MILICH D.R. & CHISARI F.V. (1982) Genetic regulation of the immune response to hepatitis B surface antigen (HBsAg). 1. H-2 restriction of the murine humoral immune response to the a and d determinants of HBsAg. *J Immunol* **129**, 320.